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Two EF-hand motifs in ryanodine receptor calcium release channels contribute to isoform-specific regulation by calmodulin

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Abstract

The mammalian ryanodine receptor Ca²⁺ release channel (RyR) has a single conserved high affinity calmodulin (CaM) binding domain. However, the skeletal muscle RyR1 is activated and cardiac muscle RyR2 is inhibited by CaM at submicromolar Ca²⁺. This suggests isoform-specific domains are involved in RyR regulation by CaM. To gain insight into the differential regulation of cardiac and skeletal muscle RyRs by CaM, RyR1/RyR2 chimeras and mutants were expressed in HEK293 cells, and their single channel activities were measured using a lipid bilayer method. All RyR1/RyR2 chimeras and mutants were inhibited by CaM at 2 μ M Ca²⁺, consistent with CaM inhibition of RyR1 and RyR2 at micromolar Ca²⁺ concentrations. An RyR1/RyR2 chimera with RyR1 N-terminal amino acid residues (aa) 1–3725 and RyR2 C-terminal aa 3692–4968 was inhibited by CaM at <1 μ M Ca²⁺ similar to RyR2. In contrast, RyR1/RyR2 chimera with RyR1 aa 1–4301 and RyR2 4254–4968 was activated at <1 μ M Ca²⁺ similar to RyR1. Replacement of RyR1 aa 3726–4298 with corresponding residues from RyR2 conferred CaM inhibition at <1 μ M Ca²⁺, which suggests RyR1 aa 3726–4298 are required for activation by CaM. Characterization of additional RyR1/RyR2 chimeras and mutants in two predicted Ca²⁺ binding motifs in RyR1 aa 4081–4092 (EF1) and aa 4116–4127 (EF2) suggests that both EF-hand motifs and additional sequences in the large N-terminal regions are required for isoform-specific RyR1 and RyR2 regulation by CaM at submicromolar Ca²⁺ concentrations.

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AUTHOR CONTRIBUTIONS

L.X., G.M., and N.Y. designed research; L.X., A.C.G., D.A.P., and N.Y. performed experiments; L.X., G.M., and N.Y. analyzed data; N.Y. and G.M. drafted manuscript. L.X., A.C.G., D.A.P., G.M., and N.Y. approved final version of manuscript.

Graphical abstract

Keywords

Excitation-contraction coupling; Skeletal muscle; Cardiac muscle; Ryanodine receptor; Ca^{2+} release channel; Calmodulin

1. INTRODUCTION

The ryanodine receptor calcium channels (RyRs) are ~2,200-kDa homotetramers that release Ca^{2+} from the sarcoplasmic reticulum to initiate cardiac and skeletal muscle contraction. Type 1 RyR (RyR1) is the dominant isoform in skeletal muscle, whereas type 2 RyR (RyR2) mediates Ca^{2+} release from cardiac muscle sarcoplasmic reticulum (1). In vitro studies showed that RyRs are regulated by a number of factors that include Ca^{2+} , ATP, and calmodulin (CaM) (2–4).

CaM is a small ubiquitously expressed 16-kDa protein that regulates multiple ion channels (5). CaM inhibits both RyR1 and RyR2 at micromolar Ca^{2+} , whereas CaM activates RyR1 and inhibits RyR2 at submicromolar Ca^{2+} (6, 7). Trypsin digestion, [^{35}S]CaM binding studies using intact RyRs, and site-directed mutagenesis demonstrated that skeletal and cardiac muscle RyRs have a single conserved high affinity CaM binding site per RyR subunit (RyR1 amino acid residues (aa) 3614–3643, RyR2 aa 3581–3610) that interacts with Ca^{2+} -free and Ca^{2+} -bound forms of CaM (8–13). Structural analysis using cryo-electron microscopy and three dimensional image analysis indicated that Ca^{2+} -free and Ca^{2+} -bound CaM bind overlapping sites distal from the channel pore, which suggests that CaM exerts its effects via interactions involving additional regions of RyR1 and RyR2 outside the CaM binding sites (14, 15). The crystal structure of the Ca^{2+} -bound CaM in complex with RyR1 3614–3643 peptide and NMR and FRET measurements suggested that the RyR1 CaM binding domain can bind both CaM lobes or only the C-lobe with the N-lobe binding to another RyR1 region (16). This could explain that studies using synthetic peptides and

fusion proteins revealed several potential CaM binding sites in RyR with variable dependence on Ca^{2+} (17–19).

Conservation of the CaM protein sequence among mammals supports its important role in cellular functions. Studies with CaM mutants and genetically modified mice showed impaired regulation of RyR by CaM alters cardiac and skeletal muscle function. Mutations in CaM were associated with severe RyR2-mediated cardiac arrhythmias (20, 21). Substitution of three amino acids in the RyR2 CaM binding domain (W3587A/L3591D/F3603A) disrupted CaM inhibition of RyR2 at diastolic and systolic Ca^{2+} concentrations and caused severe cardiac hypertrophy in mice followed by death 2–3 weeks after birth (22). An RyR1 mutant corresponding to RyR2-L3591D impaired activation by CaM at submicromolar Ca^{2+} and inhibition by CaM at micromolar Ca^{2+} , and altered skeletal muscle excitation-contraction coupling and force generation without causing lethality of the mice (23).

The CaM binding domain of RyR1 (aa 3614–3643) and RyR2 (aa 3581–3610) differ in four amino acid residues, yet substitution of these four amino acids in RyR2 with corresponding sequence from RyR1 did not change isoform specific regulation of CaM (12). This suggests that RyR regions outside the CaM binding site are involved in isoform-specific CaM regulation of RyR. To identify regions involved in differential regulation by CaM, RyR1/RyR2 chimeras and mutants expressed in HEK293 cells were analyzed for single channel activities in the presence or absence of CaM using a lipid bilayer method. The results have identified multiple sites that include the RyR1 and RyR2 EF-hand motifs required for isoform-specific regulation of RyR1 and RyR2 by CaM.

2. MATERIALS AND METHODS

2.1 Materials

Complete protease inhibitors were from Roche (Indianapolis, IN), Fugene6 from Promega (Madison, WI), jetPRIME from Polyplus-transfection (New York, NY), and human embryonic kidney (HEK) 293 cells from ATCC (Manassas, VA). Full-length wild type RyR2 and R1 chimera cDNAs (24) were kindly provided by Dr. Junichi Nakai, Saitama University, Japan. Unlabeled CaM was prepared as described (10) or purchased from Ocean Biologics (Seattle, WA).

2.2 Construction of RyR expression plasmids

Full-length rabbit RyR1 and RyR2 cDNAs were cloned into pCMV5 and pCIneo mammalian expression vectors, respectively. RyR chimeras were constructed using compatible restriction enzyme sites or by introducing new restriction enzyme sites by site-directed mutagenesis. Single and multiple base changes and deletions were introduced by *Pfu*-turbo polymerase-based chain reaction, using mutagenic oligonucleotides and QuikChange site-directed mutagenesis kit (Agilent, Santa Clara, CA). Chimeras and mutant clones were verified by DNA sequencing. Sequences and numbering are as reported (25, 26). A list of chimera and mutant RyRs are shown in Table 1. Chimeras are named in accordance to their sequences. s and c stand for skeletal (RyR1) and cardiac (RyR2) RyR

sequence, respectively, in N-terminal, EF hand motifs, and C-terminal. In some chimeras, EF hand is further separated into two domains, EF1 and EF2. Names of previously reported constructs (in parentheses) (24, 27) are *s-sEF-c* (R0), *s-cEF-c* (R1), *s-cEF-s* (R21), *s-sEF1-cEF2-s* (R41), *s-cEF1-cEF2-s* (R41'), *s-cEF1-sEF2-s* (R51), and *s-sEF1-sEF2-s* (R51'). Two chimeras highlight sequence differences in CaM binding domain (CaMBD), thus their names are *c-cCaMBD-sEF1-c* and *c-sCaMBD-sEF1-c* (Table 1).

2.3 Full-length RyR expression in HEK293 Cells

Wild type (WT) and RyR1 and RyR2 mutants were transiently expressed in HEK293 cells using Fugene6 or jetPRIME according to the manufacturer's instructions. Cells were maintained at 35 to 37°C and 5% CO₂ in high glucose Dulbecco's modified eagle medium containing 10% fetal bovine serum. Cells were transfected the day after plating and harvested after 48 hours of transfection. Membrane fractions were prepared by homogenizing cells in 0.3 M sucrose, 0.15 M KCl, 20 mM imidazole, pH 7.0, 0.1 mM EGTA, 1 mM glutathione (oxidized) and complete protease inhibitors (Roche). Homogenates were centrifuged for 45 min at 100,000 × g, and pellets were suspended in the same buffer without EGTA and glutathione.

2.4 Single Channel Analysis

Single channel measurements with wild type and mutant RyRs were performed in planar lipid bilayers (12). Membrane fractions expressing RyRs were pretreated with myosin light chain kinase CaM binding domain peptide (CaMBP) for 30 min at room temperature to remove endogenous CaM (10). Membranes were added to the cis cytosolic chamber of the bilayer apparatus. A strong dependence of single channel activities on cytosolic Ca²⁺ concentration indicated that the large cytosolic "foot" region faced the cis chamber of the bilayers. The trans sarcoplasmic reticulum luminal side of the bilayer was defined as ground. Measurements were made in symmetrical buffer containing 0.25 M KCl, 20 mM K-Hepes, pH 7.4. Activating and inhibitory effects of exogenous cis cytosolic CaM were determined at the indicated cytosolic Ca²⁺ concentrations. Electrical signals were filtered at 2 kHz, digitized at 10 kHz and analyzed (12). Open probability (P_o) was obtained from recordings lasting a minimum of 2 min. P_o values in multichannel recordings were calculated using the equation $P_o = \sum i P_{o,i} / N$, where N is the total number of channels and P_{o,i} is channel open probability of the ith channel. To obtain the number of channels incorporated into the bilayers, the number of channel current levels were determined with 2 μM cis cytosolic Ca²⁺.

2.5 Biochemical Assays and Data Analysis

Free Ca²⁺ concentrations were established using Ca²⁺ and EGTA as determined from stability constants and the algorithm of Shoenmakers *et al.* (28). Free Ca²⁺ concentrations were verified using a Ca²⁺ selective electrode. Results are indicated as the means ± SEM. Significance of CaM regulation of RyRs was determined, using Student's *t*-test or ANOVA followed by Tukey's test when comparing three groups (control (no CaM), 50 nM CaM, and 1 μM CaM). ^ p<0.05 compared with control by Student's *t*-test, and * p<0.05 and ** p<0.005 compared with control by ANOVA.

3. RESULTS

3.1 CaM regulation of RyR1/RyR2 chimeras and mutants at 2 μM Ca^{2+}

RyR1 and RyR2 are inhibited by CaM at 2 μM cytosolic Ca^{2+} in single channel measurements, whereas RyR1 is activated by CaM and RyR2 is inhibited at submicromolar Ca^{2+} (29). To identify the regions involved in this differential CaM regulation of RyR1 and RyR2 by CaM, RyR1/RyR2 chimeras and multi-site mutants were expressed in HEK293 cells (Table 1), and membrane fractions were prepared for single channel measurements. Prior to addition to the cis cytosolic bilayer chamber, membranes were treated with CaMBP to remove endogenous CaM (10). Table 1 shows that wild type, RyR1/RyR2 chimeras and mutants were significantly inhibited at 2 μM cytosolic Ca^{2+} by 50 nM and 1 μM cytosolic CaM. Furthermore, all chimera and mutant RyRs maintained Ca^{2+} -dependent [^3H]ryanodine binding comparable to that of WT-RyR1 and RyR2 (Supplementary Fig. S1) (27, 30). The results suggest that RyR1/RyR2 chimeras and mutants maintained the ability to be regulated by Ca^{2+} and bind to the Ca^{2+} -bound CaM.

3.2 CaM regulation of RyR1/RyR2 chimeras with the RyR1 N-terminal region at submicromolar Ca^{2+}

To understand how CaM regulates RyR1 and RyR2 at submicromolar cytosolic Ca^{2+} , we initially confirmed that RyR1 was activated and RyR2 was inhibited by CaM at 0.1–0.4 μM cytosolic Ca^{2+} (Fig. 1 and Table 1). The *s-cEF-c* chimera (Fig. 2A) containing RyR1 N-terminal aa 1–3725 and RyR2 C-terminal 3692–4968 was inhibited by CaM at <1 μM Ca^{2+} , similar to RyR2 (Fig. 2B). To determine the requirement for the C-terminal RyR domain, *s-sEF-c* and *s-cEF-s* chimeras were prepared (Fig. 2A). Extending the RyR1 N-terminal region by 576 amino acids in *s-sEF-c* (RyR1 aa 1–4301; RyR2 aa 4254–4968) resulted in RyR1-like CaM activation at submicromolar Ca^{2+} (Figs. 2C and 2E). The *s-cEF-s* chimera in which aa 3726–4298 of RyR1 were replaced with the corresponding RyR2 sequence, showed RyR2-type CaM inhibition at <1 μM Ca^{2+} (Figs. 2D and 2E). The results suggest that RyR1 aa 3726–4298 that correspond to RyR2 aa 3692–4250 had a role in isoform-specific CaM regulation of RyR1 and RyR2 at submicromolar Ca^{2+} .

The RyR1 CaM activation domain aa 3726–4298 contains a CaM-like domain at aa 4064–4210 that includes two EF-hand Ca^{2+} binding motifs (EF1 aa 4081–4092, and EF2 aa 4116–4127) (31). The isoform specificity of the two EF-hand motifs in CaM regulation of RyRs was investigated using four RyR1 backbone chimeras: *s-sEF1-cEF2-s* (RyR1 EF1 and RyR2 EF2), *s-cEF1-cEF2-s* (RyR2 EF1 and EF2), *s-cEF1-sEF2-s* (RyR2 EF1 and RyR1 EF2), and *s-sEF1-sEF2-s* (RyR1 EF1 and EF2) (Fig. 3A). *s-sEF1-cEF2-s* (RyR1 EF1-RyR2 EF2) was activated by CaM similar to RyR1 (Figs. 3B and 3F). In contrast, *s-cEF1-cEF2-s* (RyR2 EF1-EF2) was inhibited by CaM at submicromolar Ca^{2+} similar to RyR2 (Figs. 3C and 3F). *s-cEF1-sEF2-s* (RyR2 EF1-RyR1 EF2) lost regulation by CaM at submicromolar Ca^{2+} (Figs. 3D and 3F). *s-sEF1-sEF2-s* (RyR1 EF1-EF2) carrying RyR2 N-terminal flanking sequence (aa 3692–4019) was activated by CaM similar to RyR1 (Figs. 3E and 3F). The results suggest that in RyR1/RyR2 chimeras with RyR1 N-terminal the predicted EF1 Ca^{2+} binding region is required for RyR activation by CaM at submicromolar Ca^{2+} .

3.3 CaM regulation of EF1 and EF2 mutant RyRs at submicromolar Ca^{2+}

The results suggesting that RyR1-EF1 is required for RyR1 activation by CaM (Fig. 3) were investigated further by creating RyR1-EF1mut, in which 14 RyR1 amino acids in EF1 and flanking sequence were replaced with corresponding sequence from RyR2 (Fig. 4A). Single channel measurements showed a loss of CaM regulation at submicromolar Ca^{2+} (Fig. 4B) similar to *s-cEF1-sEF2-s* chimera (Fig. 3D).

Spectroscopic measurements and antibody studies against the RyR1 CaM binding domain (aa 3614–3643) and EF2 motif (aa 4114–4142) indicated that these two regions interact in a Ca^{2+} -dependent manner (32). To assess the role of the EF2 motif in isoform-specific CaM regulation of RyR, three EF2 mutations were created, one in RyR1 and two in RyR2 (Fig. 4A). RyR1-EF2mut (M4122T-I4123L-N4124D-F4125Y-N4130K) with five non-conserved amino acid residues in RyR1-EF2 and flanking region replaced with corresponding sequence from RyR2 (Fig. 4A), was inhibited by CaM at submicromolar Ca^{2+} (Figs. 4C and 4F). The contribution of EF2 in RyR regulation by CaM was investigated further using RyR2-EF2mut (T4077M-L4078I-D4079N-Y4080F-K4085N), the reverse mutant RyR1-EF2mut, and RyR2-EF2del that lacks four EF2 amino acid residues (Thr4077-Tyr4080) (Fig. 4A). Both RyR2-EF2mut and RyR2-EF2del were inhibited by CaM at submicromolar Ca^{2+} (Figs. 4D–F). This suggests that structural changes in EF2 suppressed RyR1 activation but not RyR2 inhibition by CaM at submicromolar Ca^{2+} . It was noted that one mutant, RyR2-EF2del, showed a high open probability at submicromolar Ca^{2+} (Fig. 4E). Open probabilities were 0.43 ± 0.19 ($n=5$), which suggests that deletion of four amino acids in EF2 altered the Ca^{2+} -dependent regulation of RyR2.

3.4 CaM regulation of RyR1/RyR2 chimeras with RyR2 N-terminal sequence at submicromolar Ca^{2+}

To investigate the requirement for the RyR2 N-terminal region, *c-cCaMBD-sEF-c* chimera was created by replacing RyR1 N-terminal aa 1–3725 in *s-sEF-c* chimera (Fig. 2) with RyR2 N-terminal aa 1–3691 (Fig. 5A). The *c-cCaMBD-sEF-c* chimera that has the RyR N-terminal regions exchanged was inhibited (Fig. 5B) rather than activated by CaM (*s-sEF-c*, Fig. 2C). The *c-cCaMBD-sEF-c* chimera had RyR2-like regulation by CaM despite two RyR1-EF-hand motifs. This suggests that the RyR1 EF-hand motifs were not sufficient to override RyR2 inhibition by CaM at submicromolar Ca^{2+} .

To determine whether the presence of an extended RyR1 region encompassing the CaM binding domain modified regulation of *c-cCaMBD-sEF-c* chimera by CaM, *c-sCaMBD-sEF-c* chimera was prepared, which differed from *c-cCaMBD-sEF-c* chimera by having RyR1 CaM binding domain (RyR1 aa 3614–3643) (Fig. 5A). The presence of the RyR1 CaM binding domain in *c-sCaMBD-sEF-c* chimera did not alter CaM inhibition at submicromolar Ca^{2+} observed for *c-cCaMBD-sEF-c* chimera (Figs. 5C and 5D).

4. DISCUSSION

This study of RyR1/RyR2 chimeras and mutants has demonstrated that two EF-hand motifs and the large N-terminal region mediate isoform-specific regulation of RyR1 and RyR2 by

CaM. Cryo-electron micrograph studies examining the architecture of the large RyR1 and RyR2 complexes have revealed the structural relationship of the high-affinity CaM binding domain (14, 15) and two EF-hand motifs (33–37). Recent high-resolution maps show that the two EF-hand motifs are near a cytoplasmic loop linking transmembrane segments S2 and S3 (34) and undergo a conformational change in the presence of Ca^{2+} with respect to the S2-S3 linker (36). Although the RyR1 high-affinity CaM binding domain (aa 3614–3643) was not resolved in the high resolution structures, lower resolution studies suggest that Ca^{2+} -free and Ca^{2+} -bound CaM bind two distinct sites near the EF-hand motifs (38). The 30 Å cryo-electron micrograph maps show the RyR2 Ca^{2+} -free CaM binding domain has a similar orientation relative to the RyR1 Ca^{2+} -bound CaM binding domain (15). While these studies provide a structural framework for Ca^{2+} -free CaM inhibition of RyR2 and Ca^{2+} -CaM inhibition of RyR1, the position of Ca^{2+} -bound CaM on RyR2 was not determined. The present study provides evidence that the two EF-hand motifs are involved in long-range ~10 nm interactions between the CaM binding domain and the channel pore region (14, 15).

The EF1 and EF2 domains are part of a CaM-like domain in RyR1 composed of ~150 amino acid residues (RyR1 aa 4064–4210) (31). A peptide corresponding to the CaM-like domain modulated RyR1 activity and bound a CaM binding domain peptide (RyR1 aa 3614–3643) in a Ca^{2+} -dependent manner. The results suggested a functional and structural interaction between the RyR1 CaM-like domain and CaM binding domain (31). Site-directed fluorescent labeling and use of domain-specific antibodies against the RyR1 CaM binding domain and 29 amino acid stretch (RyR1 aa 4114–4142) that included EF2 but not EF1 suggested that RyR1 aa 3614–3643 and 4114–4142 regions functionally interact in a Ca^{2+} -dependent manner (32).

Several lines of evidence have suggested that the two EF-hand motifs are involved in Ca^{2+} -dependent regulation of RyRs. The two EF-hand motifs in the recombinant CaM-like domain (RyR1 aa 4064–4210) bound two Ca^{2+} with an apparent affinity of 60 μM (31). A lower affinity was observed for two smaller recombinant proteins from rabbit skeletal muscle RyR1 (aa 4069–4139) and cardiac muscle RyR2 (aa 4024–4094) that bound two Ca^{2+} with apparent affinities of 3.7 mM and 3.8 mM, respectively, which suggested that sites in these regions have a role in inactivation of RyR by Ca^{2+} (39). Mutagenesis of the two EF-hand motifs in RyR1 did not reveal functional differences in response to depolarization or caffeine compared to wild type RyR1-expressing myotubes (40). However, altered Ca^{2+} activation or inactivation of EF1 mutants was observed in a binding assay using the RyR specific ligand [^3H]ryanodine. [^3H]Ryanodine binding was lost in the EF2 mutant, but Ca^{2+} -dependent activity was maintained in single channel recordings. Mutation or deletion of the two EF-hand motifs in RyR2 did not alter Ca^{2+} -dependent activation of RyR2, whereas deletion of the EF-hand motifs decreased regulation of RyR2 by luminal Ca^{2+} (41). Previously, we analyzed RyR1/RyR2 chimeras including some in the present study and determined Ca^{2+} -dependent activation or inactivation using [^3H]ryanodine (27). The *s-sEF1-cEF2-s* chimera containing RyR1-EF1 and RyR2-EF2 was only modestly affected with respect to inactivation by Ca^{2+} , whereas the *s-cEF1-sEF2-s* chimera (RyR2-EF1/RyR1-EF2) had a significantly reduced affinity of Ca^{2+} inactivation similar to RyR2 Ca^{2+} -inactivation, which suggested that EF1 is required for isoform-specific RyR inactivation by Ca^{2+} . A novel

finding of the present study is that, in addition to a role in Ca^{2+} -dependent inactivation, the EF-hand Ca^{2+} -binding motifs participate in isoform-specific regulation of RyR by CaM.

Our results showed that chimera and mutant RyRs were regulated by Ca^{2+} and inhibited by CaM at $2\mu\text{M}$ cytosolic Ca^{2+} . However, some discrepancies in their regulation by Ca^{2+} and by CaM were observed at submicromolar Ca^{2+} . Results using multiple amino acid mutations in RyR1-EF2mut that showed inhibitory effect by CaM appeared to be incompatible with results using the *s-sEF1-cEF2-s* chimera, in which replacing RyR1-EF2 and flanking region with corresponding RyR2 sequence maintained activation by CaM at submicromolar Ca^{2+} . Replacement of RyR1-EF1 with RyR2-EF1 in *s-cEF1-sEF2-s* chimera and RyR1-EF1mut with a RyR1-backbone resulted in loss of CaM regulation at submicromolar Ca^{2+} , although both were inhibited by CaM at $2\mu\text{M}$ Ca^{2+} . It should be also noted that the control P_o (no CaM) and the extent of CaM regulation varied among the constructs (Tables 2 and 3). One possible explanation may be that interaction between regions involved in CaM regulation (e.g. CaM binding domain and EF hand motifs) may be perturbed to different extent in constructs at submicromolar Ca^{2+} . A relatively high open probability of RyR2-EF2del in the absence of CaM (Fig. 4E) and low [^3H]ryanodine binding level at submicromolar Ca^{2+} (Supplementary Fig. S1) remains to be solved.

The chimera studies show that in addition to EF1 and EF2, the N-terminal regions are involved in RyR regulation by CaM. The *s-sEF-c*, *s-sEF1-cEF2-s*, and *s-sEF1-sEF2-s* chimeras with a RyR1 N-terminal region that included the RyR1-EF1 sequence were activated by CaM at submicromolar Ca^{2+} (Table 1). On the other hand, *c-cCaMBD-sEF-c* and *c-sCaMBD-sEF-c* chimeras with RyR2 N-terminal sequence were inhibited by CaM at submicromolar Ca^{2+} despite the presence of the RyR1-EF1 domain (Table 1). The results suggest that the large N-terminal regions of RyR1 (aa 1–3580) and RyR2 (aa 1–3535) are involved in the regulation of RyR-mediated Ca^{2+} release by CaM in muscle, with the RyR1 N-terminal region having a permissive role and RyR2 N-terminal region suppressing CaM activation at submicromolar Ca^{2+} . Future studies may identify additional sites within the N-terminal regions that confer isoform-specific CaM regulation of RyR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Franzini-Armstrong C, Protasi F. Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. *Physiol Rev.* 1997; 77:699–729. [PubMed: 9234963]
2. Meissner G. Regulation of mammalian ryanodine receptors. *Front Biosci.* 2002; 7:d2072–2080. [PubMed: 12438018]
3. Capes EM, Loaiza R, Valdivia HH. Ryanodine receptors. *Skelet Muscle.* 2011; 1:18. [PubMed: 21798098]

4. Lanner JT, Georgiou DK, Joshi AD, Hamilton SL. Ryanodine receptors: structure, expression, molecular details, and function in calcium release. *Cold Spring Harb Perspect Biol.* 2010; 2:a003996. [PubMed: 20961976]
5. Saimi Y, Kung C. Calmodulin as an ion channel subunit. *Annu Rev Physiol.* 2002; 64:289–311. [PubMed: 11826271]
6. Fruen BR, Bardy JM, Byrem TM, Strasburg GM, Louis CF. Differential Ca^{2+} sensitivity of skeletal and cardiac muscle ryanodine receptors in the presence of calmodulin. *Am J Physiol Cell Physiol.* 2000; 279:C724–733. [PubMed: 10942723]
7. Balshaw DM, Yamaguchi N, Meissner G. Modulation of intracellular calcium-release channels by calmodulin. *J Membr Biol.* 2002; 185:1–8. [PubMed: 11891559]
8. Moore CP, Rodney G, Zhang JZ, Santacruz-Toloz L, Strasburg G, Hamilton SL. Apocalmodulin and Ca^{2+} calmodulin bind to the same region on the skeletal muscle Ca^{2+} release channel. *Biochemistry.* 1999; 38:8532–8537. [PubMed: 10387100]
9. Rodney GG, Moore CP, Williams BY, et al. Calcium binding to calmodulin leads to an N-terminal shift in its binding site on the ryanodine receptor. *J Biol Chem.* 2001; 276:2069–2074. [PubMed: 11035044]
10. Balshaw DM, Xu L, Yamaguchi N, Pasek DA, Meissner G. Calmodulin binding and inhibition of cardiac muscle calcium release channel (ryanodine receptor). *J Biol Chem.* 2001; 276:20144–20153. [PubMed: 11274202]
11. Yamaguchi N, Xin C, Meissner G. Identification of apocalmodulin and Ca^{2+} -calmodulin regulatory domain in skeletal muscle Ca^{2+} release channel, ryanodine receptor. *J Biol Chem.* 2001; 276:22579–22585. [PubMed: 11306590]
12. Yamaguchi N, Xu L, Pasek DA, Evans KE, Meissner G. Molecular basis of calmodulin binding to cardiac muscle Ca^{2+} release channel (ryanodine receptor). *J Biol Chem.* 2003; 278:23480–23486. [PubMed: 12707260]
13. Yamaguchi N, Xu L, Pasek DA, Evans KE, Chen SRW, Meissner G. Calmodulin regulation and identification of calmodulin binding region of type-3 ryanodine receptor calcium release channel. *Biochemistry.* 2005; 44:15074–15081. [PubMed: 16274254]
14. Samso M, Wagenknecht T. Apocalmodulin and Ca^{2+} -calmodulin bind to neighboring locations on the ryanodine receptor. *J Biol Chem.* 2002; 277:1349–1353. [PubMed: 11694536]
15. Huang X, Fruen B, Farrington DT, Wagenknecht T, Liu Z. Calmodulin-binding locations on the skeletal and cardiac ryanodine receptors. *J Biol Chem.* 2012; 287:30328–30335. [PubMed: 22773841]
16. Maximciuc AA, Putkey JA, Shamoo Y, Mackenzie KR. Complex of calmodulin with a ryanodine receptor target reveals a novel, flexible binding mode. *Structure.* 2006; 14:1547–1556. [PubMed: 17027503]
17. Chen SRW, MacLennan DH. Identification of calmodulin-, Ca^{2+} -, and ruthenium red-binding domains in the Ca^{2+} release channel (ryanodine receptor) of rabbit skeletal muscle sarcoplasmic reticulum. *J Biol Chem.* 1994; 269:22698–22704. [PubMed: 7521330]
18. Guerrini R, Menegazzi P, Anacardio R, Marastoni M, Tomatis R, Zorzato F, Treves S. Calmodulin binding sites of the skeletal, cardiac, and brain ryanodine receptor Ca^{2+} channels: modulation by the catalytic subunit of cAMP-dependent protein kinase? *Biochemistry.* 1995; 34:5120–5129. [PubMed: 7711031]
19. Lau K, Chan MM, Van Petegem F. Lobe-specific calmodulin binding to different ryanodine receptor isoforms. *Biochemistry.* 2014; 53:932–946. [PubMed: 24447242]
20. Nomikos M, Thanassoulas A, Beck K, et al. Altered RyR2 regulation by the calmodulin F90L mutation associated with idiopathic ventricular fibrillation and early sudden cardiac death. *FEBS Lett.* 2014; 588:2898–2902. [PubMed: 25036739]
21. Søndergaard MT, Liu Y, Larsen KT, et al. The arrhythmogenic calmodulin p.Phe142Leu mutation impairs C-domain Ca^{2+} binding but not calmodulin-dependent inhibition of the cardiac ryanodine receptor. *J Biol Chem.* 2014; 292:1385–1395.
22. Yamaguchi N, Takahashi N, Xu L, Smithies O, Meissner G. Early cardiac hypertrophy in mice with impaired calmodulin regulation of cardiac muscle Ca^{2+} release channel. *J Clin Invest.* 2007; 117:1344–1353. [PubMed: 17431507]

23. Yamaguchi N, Prosser BL, Ghassemi F, et al. Modulation of sarcoplasmic reticulum Ca^{2+} release in skeletal muscle expressing ryanodine receptor impaired in regulation by calmodulin and S100A1. *Am J Physiol Cell Physiol*. 2011; 300:C998–C1012. [PubMed: 21289290]
24. Nakai J, Sekiguchi N, Rando TA, Allen PD, Beam KG. Two regions of the ryanodine receptor involved in coupling with L-type Ca^{2+} channels. *J Biol Chem*. 1998; 273:13403–13406. [PubMed: 9593671]
25. Nakai J, Imagawa T, Hakamata Y, Shigekawa M, Takeshima H, Numa S. Primary structure and functional expression from cDNA of the cardiac ryanodine receptor/calcium release channel. *FEBS Lett*. 1990; 271:169–177. [PubMed: 2226801]
26. Takeshima H, Nishimura S, Matsumoto T, et al. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature*. 1989; 339:439–445. [PubMed: 2725677]
27. Gomez AC, Yamaguchi N. Two regions of the ryanodine receptor calcium channel are involved in Ca^{2+} -dependent inactivation. *Biochemistry*. 2014; 53:1373–1379. [PubMed: 24521037]
28. Schoenmakers TJ, Visser GJ, Flik G, Theuvsen AP. CHELATOR: an improved method for computing metal ion concentrations in physiological solutions. *Biotechniques*. 1992; 12:870–879. [PubMed: 1642895]
29. Yamaguchi N, Xu L, Evans KE, Pasek DA, Meissner G. Different regions in skeletal and cardiac muscle ryanodine receptors are involved in transducing the functional effects of calmodulin. *J Biol Chem*. 2004; 279:36433–36439. [PubMed: 15215235]
30. Nakai J, Gao L, Xu L, Xin C, Pasek DA, Meissner G. Evidence for a role of C-terminus in Ca^{2+} inactivation of skeletal muscle Ca^{2+} release channel (ryanodine receptor). *FEBS Lett*. 1999; 459:154–158. [PubMed: 10518010]
31. Xiong L, Zhang JZ, He R, Hamilton SL. A Ca^{2+} -binding domain in RyR1 that interacts with the calmodulin binding site and modulates channel activity. *Biophys J*. 2006; 90:173–182. [PubMed: 16227507]
32. Gangopadhyay JP, Ikemoto N. Interaction of the Lys(3614)-Asn(3643) calmodulin-binding domain with the Cys(4114)-Asn(4142) region of the type 1 ryanodine receptor is involved in the mechanism of Ca^{2+} /agonist-induced channel activation. *Biochem J*. 2008; 411:415–423. [PubMed: 18171325]
33. Efremov RG, Leitner A, Aebersold R, Raunser S. Architecture and conformational switch mechanism of the ryanodine receptor. *Nature*. 2015; 517:39–43. [PubMed: 25470059]
34. Zalk R, Clarke OB, George AD, et al. Structure of a mammalian ryanodine receptor. *Nature*. 2015; 517:44–49. [PubMed: 25470061]
35. Yan Z, Bai X, Yan C, et al. Structure of the rabbit ryanodine receptor RyR1 at near-atomic resolution. *Nature*. 2015; 517:50–55. [PubMed: 25517095]
36. des Georges A, Clarke OB, Zalk R, et al. Structural basis for gating and activation of RyR1. *Cell*. 2016; 167:145–157. [PubMed: 27662087]
37. Peng W, Shen H, Wu J, et al. Structural basis for the gating mechanism of the type 2 ryanodine receptor RyR2. *Science*. 2016; 354 aah5324.
38. Yuchi J, Van Petegem F. Ryanodine receptor under the magnifying lens: Insights and limitation of cryo-electron microscopy and X-ray crystallography studies. *Cell Calcium*. 2016; 59:209–227. [PubMed: 27103405]
39. Xiong H, Feng X, Gao L, et al. Identification of a two EF-hand Ca^{2+} binding domain in lobster skeletal muscle ryanodine receptor/ Ca^{2+} release channel. *Biochemistry*. 1998; 37:4804–4814. [PubMed: 9537997]
40. Fessenden JD, Feng W, Pessah IN, Allen PD. Mutational analysis of putative calcium binding motifs within the skeletal ryanodine receptor isoform, RyR1. *J Biol Chem*. 2004; 279:53028–53035. [PubMed: 15469935]
41. Guo W, Sun B, Xiao Z, et al. The EF-hand Ca^{2+} binding domain is not required for cytosolic Ca^{2+} activation of the cardiac ryanodine receptor. *J Biol Chem*. 2016; 291:2150–2160. [PubMed: 26663082]

Highlights

- Calmodulin (CaM) activates RyR1 and inhibits RyR2 at submicromolar Ca^{2+} .
- RyR1/RyR2 chimera channels were generated to identify regions involved in isoform-specific CaM regulation of RyR.
- Two EF-hand motifs and a large N-terminal region are involved in isoform-specific CaM regulation of RyRs.

Figure 1. Calmodulin regulation of WT-RyR1 and WT-RyR2

(A and B) Membrane fractions pretreated with CaMBP were added to the cis cytosolic chamber of the bilayer apparatus and single channels of WT-RyR1 and WT-RyR2 were recorded at -20 mV holding potential and $0.1\text{--}0.4$ μ M cytosolic Ca^{2+} in the absence or presence of 50 nM and 1 μ M CaM. Downward deflections indicate channel openings. (C) Open probability of WT-RyR1 and WT-RyR2 were normalized to the open probabilities in the absence of CaM (control) and are shown as mean $\pm$ SEM. Number of experiments are shown on each bar. ^ $p < 0.05$ compared with controls (no CaM addition) by Student's t -test. * $p < 0.05$ and ** $p < 0.005$ compared with controls by ANOVA followed by Tukey's test.

Figure 2. Calmodulin regulation of RyR1/RyR2 chimeras

(A) Schematic of wild type and four RyR chimeras. Red and blue indicate the positions of CaM binding domain and CaM-like domain, respectively. (B–D) Membrane fractions containing *s-cEF-c* (B), *s-sEF-c* (C), and *s-cEF-s* (D) chimeras were added to cis cytosolic chamber of the bilayer apparatus. Channels were recorded at 0.1–0.4 μ M Ca^{2+} in the presence or absence of 50 nM and 1 μ M CaM. Downward deflections indicate channel openings. (E) Open probability of chimera channels in the presence of 50 nM (open bars) and 1 μ M (filled bars) CaM were normalized to the open probabilities in the absence of CaM (control) and are shown as mean $\pm$ SEM. Number of experiments are shown on each bar. ^

$p < 0.05$ compared with controls (no CaM addition) by Student's t -test. * $p < 0.05$ compared with controls by ANOVA followed by Tukey's test.

Figure 3. Calmodulin regulation of RyR1/RyR2 chimeras carrying different regions of CaM-like domain

(A) Schematic of RyR chimeras and the position of CaM-like domain (CaMLD). (B–E) Membrane fractions containing RyR chimeras were added to the cis cytosolic chamber of the bilayer apparatus and single channels were recorded at 0.1–0.4 μM Ca^{2+} in the presence or absence of 50 nM and 1 μM CaM for *s-sEF1-cEF2-s* (B), *s-cEF1-cEF2-s* (C), *s-cEF1-sEF2-s* (D), and *s-sEF1-sEF2-s* (E) chimeras. Downward deflections indicate channel openings. (F) Open probability of chimera channels in the presence of 50 nM (open bars) and 1 μM (filled bars) CaM are normalized to the open probabilities in the absence of CaM (control) and are shown as mean $\pm$ SEM. Number of experiments are shown on each bar.

Data for *s-cEF-s* chimera are the same as in Fig. 2E. [^] $p < 0.05$ compared with controls (no CaM addition) by Student's *t*-test. * $p < 0.05$ and ** $p < 0.005$ compared with controls by ANOVA followed by Tukey's test.

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Figure 4. Calmodulin regulation of EF-hand RyR mutants

(A) Sequences of EF1 and EF2 and flanking regions of wild type and mutant RyRs. Blue denotes non-conserved amino acid residues, and red amino acids were mutated. (B–E) Membrane fraction containing RyR1-EF1mut (B), RyR1-EF2mut (C), RyR2-EF2mut (D), and RyR2-EF2del (E) were added to the cis cytosolic chamber of the bilayer apparatus. Single channels were recorded at 0.1–0.4 μM Ca^{2+} in the presence or absence of 50 nM and 1 μM CaM. Downward deflections indicate channel openings. (F) Open probability of chimera channels in the presence of 50 nM (open bars) and 1 μM (filled bars) CaM are normalized to the open probabilities in the absence of CaM (control) and are shown as mean

± SEM. Number of experiments are shown on each bar. ^ p<0.05 compared with controls (no CaM addition) by Student's *t*-test. * p<0.05 and ** p<0.005 compared with controls by ANOVA followed by Tukey's test.

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Figure 5. Calmodulin regulation of RyR2-backbone chimeras

(A) Schematic of R0, R22, and R32 chimeras. (B and C) Membrane fractions containing *c-cCaMBD-sEF-c* (B) and *c-sCaMBD-sEF-c* (C) chimeras were added to the cis cytosolic chamber of the bilayer apparatus and single channels were recorded at 0.1–0.4 μM Ca^{2+} in the presence or absence of 50 nM and 1 μM CaM. Downward deflections indicate channel openings. (D) Open probability of three chimera channels in the presence of 50 nM (open bars) and 1 μM (filled bars) CaM are normalized to the open probabilities in the absence of CaM (control) and are shown as mean $\pm$ SEM. Number of experiments are shown on each bar. Data for *s-sEF-c* chimera shown as references are from Fig. 2E. ^ $p < 0.05$ compared with controls (no CaM addition) by Student's *t*-test. * $p < 0.05$ and ** $p < 0.005$ compared with controls by ANOVA followed by Tukey's test.

Table 1

List of chimera and mutant RyRs

Amino Acids	N-terminal 1–3580	CaMBD 3581–3725	EF1 4065–4097	EF2 4098–4299	C-terminal 4300–5038	Type of regulation
WT-RyR1	RyR1	RyR1	RyR1	RyR1	RyR1	activation
s-cEF-c (R1)	RyR1	RyR1	RyR2	RyR2	RyR2	inhibition
s-sEF-c (R0)	RyR1	RyR1	RyR1	RyR1	RyR2	activation
s-cEF-s (R21)	RyR1	RyR1	RyR2	RyR2	RyR1	inhibition
s-sEF1-cEF2-s (R41)	RyR1	RyR1	RyR1	RyR2	RyR1	activation
s-cEF1-cEF2-s (R41')	RyR1	RyR1	RyR2	RyR2	RyR1	inhibition
s-cEF1-sEF2-s (R51)	RyR1	RyR1	RyR2	RyR1	RyR1	loss of activation
s-sEF1-sEF2-s (R51')	RyR1	RyR1	RyR1	RyR1	RyR1	activation
RyR1-EF1mut	RyR1	RyR1	RyR2	RyR1	RyR1	loss of activation
RyR1-EF2mut	RyR1	RyR1	RyR1	modified RyR1	RyR1	inhibition
WT-RyR2	RyR2	RyR2	RyR2	RyR2	RyR2	inhibition
RyR2-EF2mut	RyR2	RyR2	RyR2	modified RyR2	RyR2	inhibition
RyR2-EF2del	RyR2	RyR2	RyR2	modified RyR2	RyR2	inhibition
c-cCaMBD-sEF-c	RyR2	RyR2	RyR1	RyR1	RyR2	inhibition
c-sCaMBD-sEF-c	RyR2	RyR1	RyR1	RyR1	RyR2	inhibition

Names of chimera are according to type of RyR sequence (s; RyR1 and c; RyR2) for N terminal-EF1-EF2-C terminal except two RyR2 backbone chimeras, for which type of CaMBD sequence is also included. Amino acid numbers correspond to those of RyR1

Table 2CaM regulation of wild type and mutant RyRs at 2 μM Ca^{2+}

	Open Probability at 2 μM Ca^{2+}	Normalized Open Probability at 2 μM Ca^{2+} (% no CaM)	
	no CaM	50 nM CaM	1 μM CaM
WT-RyR1	0.06 $\pm$ 0.01 (4)	58 $\pm$ 10 ^{**} (4)	45 $\pm$ 7 ^{**} (4)
WT-RyR2	0.45 $\pm$ 0.18 (4)	76 $\pm$ 5 [*] (4)	54 $\pm$ 7 ^{**} (4)
s-cEF-c	0.44 $\pm$ 0.05 (5)	42 $\pm$ 19 [*] (5)	40 $\pm$ 16 [*] (5)
s-sEF-c	0.31 $\pm$ 0.05 (6)	69 $\pm$ 4 ^{**} (6)	48 $\pm$ 9 ^{**} (6)
s-cEF-s	0.22 $\pm$ 0.09 (6)	52 $\pm$ 16 [^] (6)	45 $\pm$ 20 [*] (6)
s-sEF1-cEF2-s	0.22 $\pm$ 0.08 (4)	38 $\pm$ 9 ^{**} (4)	14 $\pm$ 6 ^{**} (4)
s-cEF1-cEF2-s	0.58 $\pm$ 0.04 (4)	65 $\pm$ 7 [*] (4)	38 $\pm$ 13 ^{**} (4)
s-cEF1-sEF2-s	0.25 $\pm$ 0.02 (5)	69 $\pm$ 9 [*] (5)	54 $\pm$ 9 ^{**} (5)
s-sEF1-sEF2-s	0.07 $\pm$ 0.02 (4)	51 $\pm$ 1 ^{**} (4)	50 $\pm$ 8 ^{**} (4)
RyR1-EF1mut	0.27 $\pm$ 0.03 (4)	48 $\pm$ 4 ^{**} (4)	45 $\pm$ 9 ^{**} (4)
RyR1-EF2mut	0.20 $\pm$ 0.01 (4)	35 $\pm$ 11 ^{**} (4)	24 $\pm$ 9 ^{**} (4)
RyR2-EF2mut	0.45 $\pm$ 0.09 (5)	68 $\pm$ 10 [^] (5)	58 $\pm$ 14 [*] (5)
RyR2-EF2del	0.40 $\pm$ 0.10 (4)	74 $\pm$ 9 [*] (4)	78 $\pm$ 8 [^] (4)
c-cCaMBD-sEF-c	0.38 $\pm$ 0.08 (5)	49 $\pm$ 15 [*] (5)	41 $\pm$ 11 [*] (5)
c-sCaMBD-sEF-c	0.48 $\pm$ 0.03 (5)	75 $\pm$ 5 ^{**} (5)	65 $\pm$ 3 ^{**} (5)

Data are shown as mean $\pm$ SEM of the number of experiments indicated in parentheses. All experiments were performed with membrane fractions pretreated with CaMBP. CaM inhibition effects were normalized to the control (no CaM).

[^] p<0.05 compared with controls (no CaM addition) by Student's *t*-test.

^{*} p<0.05 and

^{**} p<0.005 compared with controls by ANOVA followed by Tukey's test.

Table 3

CaM regulation of wild type and mutant RyRs at 0.1–0.4 μM Ca^{2+}

	Open Probability at 0.1–0.4 μM Ca^{2+}	Normalized Open Probability at 0.1–0.4 μM Ca^{2+} (% no CaM)	
	no CaM	50 nM CaM	1 μM CaM
WT-RyR1	0.02 $\pm$ 0.01 (6)	175 $\pm$ 22 [^] (6)	255 $\pm$ 32 ^{**} (6)
WT-RyR2	0.03 $\pm$ 0.02 (6)	57 $\pm$ 12 ^{**} (6)	53 $\pm$ 7 ^{**} (5)
s-cEF-c	0.03 $\pm$ 0.01 (7)	58 $\pm$ 17 [*] (7)	51 $\pm$ 10 [*] (7)
s-sEF-c	0.01 $\pm$ 0.01 (7)	192 $\pm$ 41 [^] (7)	353 $\pm$ 78 [*] (7)
s-cEF-s	0.01 $\pm$ 0.01 (8)	85 $\pm$ 12 (8)	58 $\pm$ 8 [*] (8)
s-sEF1-cEF2-s	0.04 $\pm$ 0.02 (4)	166 $\pm$ 25 [^] (4)	191 $\pm$ 21 [*] (4)
s-cEF1-cEF2-s	0.07 $\pm$ 0.02 (5)	66 $\pm$ 7 [*] (5)	56 $\pm$ 9 ^{**} (5)
s-cEF1-sEF2-s	0.04 $\pm$ 0.01 (4)	107 $\pm$ 11 (4)	98 $\pm$ 10 (4)
s-sEF1-sEF2-s	0.02 $\pm$ 0.01 (9)	127 $\pm$ 20 (9)	167 $\pm$ 23 [*] (9)
RyR1-EF1mut	0.01 $\pm$ 0.01 (5)	114 $\pm$ 22 (5)	94 $\pm$ 7 (5)
RyR1-EF2mut	0.06 $\pm$ 0.01 (4)	35 $\pm$ 13 ^{**} (4)	21 $\pm$ 9 ^{**} (4)
RyR2-EF2mut	0.03 $\pm$ 0.01 (6)	43 $\pm$ 12 ^{**} (6)	38 $\pm$ 9 ^{**} (6)
RyR2-EF2del	0.43 $\pm$ 0.19 (5)	84 $\pm$ 9 (5)	61 $\pm$ 15 [^] (5)
c-cCaMBD-sEF-c	0.12 $\pm$ 0.07 (4)	66 $\pm$ 10 ^{**} (4)	65 $\pm$ 3 ^{**} (4)
c-sCaMBD-sEF-c	0.08 $\pm$ 0.03 (4)	71 $\pm$ 8 [*] (4)	52 $\pm$ 9 ^{**} (4)

Data are shown as mean $\pm$ SEM of the number of experiments indicated in parentheses. All experiments were performed with membrane fractions pretreated with CaMBP. CaM activation and inhibition effects were normalized to the control (no CaM).

[^] p<0.05 compared with controls (no CaM addition) by Student's *t*-test.

^{*} p<0.05 and

^{**} p<0.005 compared with controls by ANOVA followed by Tukey's test